Superoxide and Peroxynitrite Inactivate Aconitases, but Nitric Oxide Does Not*

Alfred Hausladen† and Irwin Fridovich§
From the Department of Biochemistry, Duke University Medical Center, Durham, North Carolina 27710

The Escherichia coli and recombinant human cytosolic aconitases are inactivated by O2, with a rate constant of $-3 \times 10^4 \text{M}^{-1}\text{s}^{-1}$; the corresponding value for the porcine mitochondrial aconitase is $-0.8 \times 10^4 \text{M}^{-1}\text{s}^{-1}$. Nitric oxide, which is reported to inactivate aconitase, did not do so at a perceptible rate, while incubation with peroxynitrite led to a rapid loss of aconitase activity. We propose that the reported inactivation of aconitase by nitric oxide in vivo is actually mediated through peroxynitrite, the product of the reaction between O2 and NO.

Several dehydratases, which bear [4Fe-4S] prosthetic groups, are inactivated by O2, and this seems to have physiological consequences (1-10). Thus, the dihydroxy-acid dehydratase from aerobic E. coli was progressively inactivated when protein synthesis was blocked, and this was reversed under anaerobic conditions (1). Compounds that increase intracellular O2 production augmented the aerobic inactivation, as did hyperbaric oxygen (10). The aconitase from E. coli was similarly inactivated during exposure to peroxynitrite and was restored under anaerobiosis (8).

These effects are explained by oxidation of susceptible [4Fe-4S] clusters by O2, with concomitant loss of Fe(II) (9). Reactivation should then require both free Fe(II) and a reductant. The need for free Fe(II) had been shown by the blocking effect of a.o'-dipyridyl (8).

There are other examples of the physiological consequences of the sensitivity of [4Fe-4S]-containing dehydratases to O2. Thus, conditions that increased intracellular production of O2 imposed an auxotrophy for branched chain amino acids, due to inactivation of the dihydroxy-acid dehydratase (1, 11-13); decreased the growth rate on gluconate, due to inactivation of 6-phosphogluconate dehydratase (6); and diminished growth on succinate, due to inactivation of aconitase (7).

These dehydratases are clearly important intracellular targets for O2; yet, there has been considerable variability in the values reported for the pertinent rate constants. For the O2/6-phosphogluconate dehydratase, $k$ was estimated to be $2 \times 10^3 \text{M}^{-1}\text{s}^{-1}$ (6), and for the O2/aconitase, a value of $2 \times 10^6 \text{M}^{-1}\text{s}^{-1}$ was reported (7). In contrast to these results, in which cell extracts were used, rate constants in the range of $1-6 \times 10^6 \text{M}^{-1}\text{s}^{-1}$ were reported when the isolated enzymes were studied (9).

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† R. J. Reynolds-Leon Golberg Postdoctoral Fellow.
‡ To whom correspondence should be addressed. Tel.: 919-684-5122; Fax: 919-684-8885.
§ The abbreviations used are: SOD, superoxide dismutase; IREBP, iron-responsive element-binding protein.

There is an obvious, yet previously unrecognized source of error that arises when competitive kinetic methods are applied to extracts. Thus, the SOD1 used to compete for O2 was competing not only with the enzyme being followed, but also with the sum of all targets for O2 in the extract. The concentration of SOD needed to halve the rate of inactivation of the chosen target in the extract will therefore be greater than would be the case for that same target in isolation. This leads to overestimation of the rate constant for the O2/target reaction. It was incumbent upon us to re-examine the rate constants for the reaction of O2 with several [4Fe-4S]-containing dehydratases. Since NO has also been reported to inactivate such enzymes (14, 15) and since NO reacts with O2 at a diffusion-limited rate, yielding peroxynitrite (16), it was also important to explore the effect of NO2 on conditions that preclude the involvement of O2 and the effect of ONOO−.

EXPERIMENTAL PROCEDURES

Materials—Isocitrate, hypoxanthine, betaine, and porcine mitochondrial aconitase were from Sigma. Catalase was purchased from Boehringer Mannheim and was purified from contaminating CuZnSOD by gel filtration on a Superose 12 HR 16/50 column (Pharmacia Biotech Inc.) in 50 mM Tris-HCl, pH 8.0, containing 0.1 mM EDTA. Tris was from ICN Biochemicals; sodium citrate, sodium chloride, magnesium sulfate, EDTA, and ammonium sulfate were from Mallinckrodt Specialty Chemicals; and CuZnSOD was from Diagnostica Data Inc. Xanthine oxidase was purified according to the method of Waud et al. (17). A saturated solution of nitric oxide was produced by bubbling authentic NO gas through oxygen-free water in a sealed vial. Peroxynitrite was synthesized with a three-syringe pump from HNO3 and H2O2 according to the method of Reed et al. (18), as outlined by Radi et al. (19). Human recombinant iron-responsive element-binding protein (also called cytosolic aconitase or iron-responsive factor) was kindly provided by Drs. Tracey Rouault and Richard Klausner (NICHD, National Institutes of Health, Bethesda, MD) and M. C. Kennedy and H. Beinert (Medical College of Wisconsin, Milwaukee, WI).

Purification of Aconitase—The purification procedure was a modification of the method described by Prodromou et al. (20). An 8-liter culture of E. coli AB1157 was grown to late-log phase in LB medium at 37 °C under vigorous aeration. Cells were harvested by centrifugation and washed once in 20 mM Tris-HCl, pH 7.6, containing 1 mM citrate, 1 mM cysteine, and 0.5 mM MnCl2 (TCM buffer). Homogenization was performed in the same buffer by passage through a French pressure cell. The homogenate was fractionated with (NH4)2SO4, and the protein precipitating between 35 and 75% saturation was redissolved in 50 ml 10 mM citrate in TCM buffer. The solution was applied to a 38 x 2.6-cm phenyl-Sepharose Fast Flow column (Pharmacia Biotech Inc.) and eluted with a linear gradient of 1 M (NH4)2SO4. The solution was applied to a 38 x 2.6-cm phenyl-Sepharose Fast Flow column (Pharmacia Biotech Inc.) and eluted with a linear gradient of 1 to 0 M (NH4)2SO4 in TCM buffer. Fractions containing aconitase activity were concentrated and desalted in a pressurized cell over a PM-30 membrane (Amicon, Inc.). The protein was then applied to a 22 x 2.1-cm Procion Red column (Amicon, Inc.), and the column was washed with 50 ml of 10 mM citrate in TCM buffer. The protein was concentrated as described above and applied to a Superose 12 HR 16/50 column equilibrated in TCM buffer containing 0.2 M NaCl and 10% (w/v)

1 The abbreviations used are: SOD, superoxide dismutase; IREBP, iron-responsive element-binding protein.
betaine. Fractions containing aconitase activity were pooled and concentrated to 1 mg/ml protein by ultrafiltration.

**Assays**—Aconitase activity was measured as the formation of cis-aconitate from isocitrate at 240 ms (21) in 50 mM Tris-HCl, pH 7.4, containing 20 mM isocitrate and 0.1 mM MnCl₂. One unit is defined as the amount of enzyme necessary to produce 1 pmol of cis-aconitate/min at 20°C. Xanthine oxidase (22) and SOD (23) were measured as described, and catalase was measured according to Chance and Maehly (24). Protein was determined by the method of Bradford (25) using the Bio-Rad protein assay kit.

**Activation of Aconitase**—All solutions were prepared anaerobically from preweighed chemicals in a glove box under an atmosphere of 5% CO₂, 10% H₂, and 85% N₂. Partially active or inactive aconitase was activated by adding the following to 90 μl of a 10 μM enzyme solution: 10 μl of 0.5 mM dithiothreitol, 1 ml of 20 mM Na,S, and 1 ml of 20 mM ferrous ammonium sulfate in 50 mM Tris-HCl, pH 8.0. The activated enzyme was stored at -70°C until used for analysis. In this form, aconitase activity was stable for at least 2 weeks. Before use, aconitase was thawed on ice and desalted using an FPLC Fast Desalting HR 10/10 column (Pharmacia Biotech Inc.) equilibrated in 50 mM Tris-HCl containing 0.1 mM EDTA. The pH was 8.0 for experiments with superoxide and nitric oxide and 7.4 for experiments with peroxynitrite.

**Inactivation of Aconitases by Superoxide**—Inactivation of E. coli aconitase, porcine mitochondrial aconitase, or human recombinant IREBP by superoxide was performed in 100 mM reaction mixtures of 0.1–0.1 mM enzyme, 0.2 mM hypoxanthine, 500 units/ml catalase, and 12 μm xanthine oxidase. When indicated, the solution also contained 0.78–12.5 μM SOD or 2–5 μM citrate. At the indicated time intervals, aliquots of the solution were removed and assayed for aconitase activity. The 50-fold dilution and the Mn⁺⁺ contained in the assay buffer essentially stopped the reaction of O₂⁻ with the aconitases.

**Incubation of Aconitase with Nitric Oxide**—E. coli aconitase or human recombinant IREBP was incubated with nitric oxide in anaerobic Hungate tubes for 30 min at room temperature. These rubber tubes, which also sealed the reaction vessels, contained 50 mM Tris-HCl, pH 8.0, 0.1 mM EDTA, and 0.6 μM aconitase, were prepared in an anaerobic glove box. Additions of nitric oxide from a saturated anaerobic solution in H₂O, was not a factor since H₂O₂ was not a factor since 500–5000 units/ml catalase did not significantly influence the inactivation of the E. coli aconitase. If O₂⁻ attacks iron of the cluster that lacks a cysteine-S-ligand and that is presumed to interact with substrate, then nitrate should protect aconitase against O₂⁻. The data in Fig. 3 show that it did so. Since most intracellular citrate is bound to divalent cations, we investigated the effect of magnesium on the protection of mitochondrial aconitase against O₂⁻ by citrate. Table II shows that mitochondrial aconitase was similarly protected by 10 μM citrate compared with the E. coli enzyme, but the protection was

![Figure 1](image-url)

**RESULTS**

**Inactivation of Aconitase by O₂⁻**—When pure aconitase was exposed to a flux of O₂⁻, produced by the xanthine oxidase reaction, it was progressively inactivated, and SOD decreased the rate of inactivation. This was the case for the E. coli and human cytosolic aconitases. The porcine mitochondrial aconitase gave similar results. The rate of inactivation of E. coli aconitase at a constant flux of O₂⁻ was a first-order dependent of the residual [aconitase], and this is shown, for several SOD concentrations, in Fig. 1A. The slopes of these first-order plots were, in turn, linearly related to the [SOD] present, as shown in Fig. 1B. Comparable data for the human cytosolic aconitase (or IREBP) are shown in Fig. 2.

The concentrations of SOD needed to halve the rate of inactivation of given concentrations of these aconitases, as well as the rate constants calculated for their reactions with O₂⁻, are given in Table I. For the E. coli aconitase, k = 3 x 10⁻⁷ M⁻¹ s⁻¹. The human cytosolic aconitase was comparably sensitive toward O₂⁻, whereas the porcine mitochondrial enzyme was more resistant (k = 0.8 x 10⁻⁷ M⁻¹ s⁻¹). H₂O₂ was not a factor since 500–5000 units/ml catalase did not significantly influence the inactivation of the E. coli aconitase. If O₂⁻ attacks iron of the cluster that lacks a cysteine-S-ligand and that is presumed to interact with substrate, then citrate should protect aconitase against O₂⁻. The data in Fig. 3 show that it did so. Since most intracellular citrate is bound to divalent cations, we investigated the effect of magnesium on the protection of mitochondrial aconitase against O₂⁻ by citrate. Table II shows that mitochondrial aconitase was similarly protected by 10 μM citrate compared with the E. coli enzyme, but the protection was
Oi, NO, and Aconitases

Fig. 2. First-order plot of the initial rate of inactivation of 0.88 μM human recombinant IREBP by O₂. A, incubation was as described in the legend to Fig. 1 with O (○), 0.78 (●), 1.56 (▲), 3.12 (▽), 6.25 (□), and 12.5 (▲) nM SOD. B, secondary plot of the initial rates of inactivation of E. coli aconitase by O₂.

Table I: Rate constants for the reaction of aconitases with O₂

<table>
<thead>
<tr>
<th>Aconitase</th>
<th>SOD needed for 50% protection (μM)</th>
<th>Rate constant (k) (s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli</td>
<td>0.19 1.6 2.6 x 10⁷</td>
<td></td>
</tr>
<tr>
<td>IREBP</td>
<td>0.14 1.6 3.4 x 10⁷</td>
<td></td>
</tr>
<tr>
<td>Mitochondrial</td>
<td>8.3 22.5 8.1 x 10⁹</td>
<td></td>
</tr>
</tbody>
</table>

* Aconitase was incubated with 500 units of SOD-free catalase (Boehringer Mannheim) in a 0.1-ml reaction volume.

Fig. 3. Protection of 0.25 μM E. coli aconitase by citrate from inactivation by O₂. Shown is the first-order plot of the initial rates of inactivation in the presence of 0 (○), 5 (●), 10 (▲), and 20 (▽) μM citrate. Reaction conditions were as described in the legend to Fig. 1.

Table II: Effect of citrate and magnesium on the inactivation of mitochondrial aconitase by O₂

<table>
<thead>
<tr>
<th>Citrate</th>
<th>Mg²⁺</th>
<th>t₁/₂ (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>μM</td>
<td>mm</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>0</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>10</td>
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<td>20</td>
</tr>
<tr>
<td>10</td>
<td>1</td>
<td>8</td>
</tr>
<tr>
<td>10</td>
<td>5</td>
<td>6</td>
</tr>
</tbody>
</table>

*Errors associated with the second-order regression lines used to calculate the rate constants were generally below 20%.

Results are given for three independent experiments.

diminished by increasing concentrations of Mg²⁺ and was eliminated by [Mg²⁺] below that which pertained in vivo.

Nitric Oxide Does Not Inactivate Aconitase—As shown in Fig. 4, E. coli aconitase was rapidly inactivated by ferricyanide, as expected (27); however, it was not affected by NO⁻ in the absence of dioxygen. Aerobic NO⁻ exerted only a modest effect, as did nitrite. Similar results were obtained for cytosolic aconitase. NO⁻ produced in vivo may well be converted to peroxynitrite because of its rapid reaction with O₂ (16). Peroxynitrite was tested accordingly and found to rapidly inactivate E. coli aconitase, as shown in Fig. 5. It should be noted that these exposures to peroxynitrite were for 15 s, during which time [ONOO⁻] was decreasing due to its intrinsic instability at neutral pH. The ONOO⁻ concentrations shown in Fig. 5 are thus initial concentrations. It follows that inactivation of aconitase by ONOO⁻ is a rapid process.
The E. coli and human cytosolic aconitases were inactivated by O$_3^-$ with a rate constant close to 3 x 10$^{-3}$ m$^{-1}$ s$^{-1}$. This is a factor of 10 greater than that reported by Flint et al. (9) for beef heart aconitase and is 100-fold less than we previously measured with extracts of a SOD-null strain of E. coli (7, 8). It is certain that cell extracts contain multiple targets for O$_3^-$ besides aconitase. Our earlier failure to consider that the SOD added to the assay mixture was competing for O$_3^-$ with the totality of such targets, rather than only with the target we were measuring, accounts for the very high rate constants previously reported (6–8).

The cytosolic aconitase also reacted with O$_3^-$, with $k = 3 \times 10^{-3}$ m$^{-1}$ s$^{-1}$, and this may be a factor in its function as a translational regulator of the biosynthesis of ferritin and the transferrin receptor. Thus, the balance between the iron-replete and the transferrin receptor biosynthesis.

**REFERENCES**


3 R. Radi, personal communication.